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The rotation of the α subunit of F_1 relative to minor subunits is not involved in ATP synthesis. Evidence given by using an anti-α subunit monoclonal antibody

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To test whether ATP synthesis could occur via a mechanism of rotational catalysis in which the α and β subunits of F_1 would rotate with respect to the minor subunits, we have measured the rate of ATP synthesis after binding various masses of antibodies to F_1. If the rotation was an essential feature of the mechanism, the rate of ATP synthesis should be inhibited either completely or proportionately to the load carried by F_1. Bivalent immunoglobulins (IgG) or monovalent Fab fragments of an anti-α monoclonal antibody ($7B_3$) were bound to F_1 present in electron-transport particles in a ratio of 2 Fab or 2 IgG per F_1. This binding similarly inhibited the rate of ATP synthesis by a maximum of about 50%. When anti-mouse immunoglobulins were added to the F_1-$7B_3$ (IgG) complex, no significant change in the rate of inhibition was observed. In conclusion, the rate of ATP synthesis was the same when F_1 was loaded with 100 kDa (2 Fab), 300 kDa (2 IgG, $7B_3$) or 900 kDa (2 IgG + 4 anti-mouse IgG). It is concluded that the rotation of the α subunits is extremely unlikely to play an essential role in the mechanism of ATP synthesis.

The mitochondrial ATPase-ATP synthase (F_0-F_1) catalyzes ATP synthesis during oxidative phosphorylations. In mitochondria, as well as in bacteria and chloroplasts, this complex is made of two parts, a membrane part, F_0, forming a proton channel and a hydrophilic part, F_1, which contains the catalytic sites of ATP synthesis and hydrolysis. F_1 is made of five subunits: α, β, γ, δ and ϵ. There are three copies of the α and β subunits and one copy of the γ, δ and ϵ subunits (for recent reviews, see Refs. 1–5). Recent structural studies [6,7] are in agreement with the asymmetrical arrangement of the α and β subunits proposed by Amzel and Pedersen [1], one of the α-β couples being tagged by the minor subunits [3,6,7]. Chemical modification studies, kinetic data and nucleotide binding experiments also demonstrate a functional heterogeneity of the α and β subunits [3,8–11]. The enzyme contains 6 nucleotide binding sites [12] located on the β subunits and/or at α–β interfaces [1–3,13–15]. There is a controversy as to how many of the sites are catalytic or regulatory [3,8,11,16–18].

Boyer [19] has proposed a model for the mechanism of ATP synthesis, defined as 'rotational catalysis' in which three equivalent catalytic sites function alternatively. In this model, a functional asymmetry is created by a rotation in the position of the α/β subunits relative to the minor subunits γ, δ, ϵ occurring during ATP synthesis or hydroly-

Abbreviations: P_i, inorganic phosphate; PBS, phosphate-buffered saline.

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sis. Cox et al. [20,21] have further developed this model, involving F_0 subunits in the rotation.

This mechanism of rotational catalysis is different from the rapid rotational diffusion of F_1 in solution [22,23] and from the rapid lateral or rotational diffusion of F_0F_1 in lipid bilayers. Indeed such movements have been described for other membrane proteins, i.e., cytochrome *P*-450 in microsomes [24] or electron-transfer enzyme complexes in inner mitochondrial membranes [25]. However, this rotational diffusion corresponds to a rotation of the whole F_1 or F_0F_1 molecule and is not related to the mechanism of ATP synthesis. On the contrary, the rotational catalysis implies a friction of the larger subunits α and β with the minor ones inside the F_1 or F_0F_1 complex. The latter type of rotation must occur at the rate of ATP hydrolysis or synthesis.

To prove or disprove this rotational catalysis mechanism, the enzymatic activity has been studied while the polypeptide chain rotation was prevented by cross-linking the α or β subunits with the minor ones. Using this approach, two contradictory conclusions have been reported. According to Kandpal and Boyer [26], "catalysis by the *Escherichia coli* F_1-ATPase is highly sensitive to the restrictions of crosslinking". This result is consistent with the model of rotational catalysis. On the contrary, the results obtained by Musier and Hammes [27] on chloroplast F_1-ATPase indicate that "large movements of α and β polypeptides with respect to the γ polypeptide are not essential for catalysis". In addition, Wang et al. [28] have been able to distinguish the three β subunits of mitochondrial F_1 by specific labeling of either the hydrolytic or non-hydrolytic β subunits with 7-chloro-4-nitro-2,1,3 benzoxadiazole (NBD-Cl). Although the labeling at the non-hydrolytic β subunits did not inhibit ATP hydrolysis in reconstituted submitochondrial particles, this labeling inhibited oxidative phosphorylation. They concluded that the two types of β subunit (hydrolytic or not) did not switch roles during oxidative phosphorylations and therefore that a rotation of the β subunits relative to γ δ ϵ did not occur during ATP synthesis in reconstituted submitochondrial particles.

The availability of monoclonal antibodies in our laboratory [29,30] has permitted us to use a different approach: in the present work, the rate of ATP synthesis has been studied in submitochondrial particles after loading the α subunit of F_1 with monoclonal antibodies (mAb) or antibody complexes of various mass. The rationale of these experiments was the following: if the rotation of α and β subunits is the factor controlling the rate of catalysis, the binding of heavy mass to the subunits should decrease their rate of rotation. Therefore, the rate of ATP synthesis should either be decreased proportionally to the size of the load or be completely abolished. ATP synthesis was studied rather than ATP hydrolysis, since it is the essential function of the ATPase-ATP synthase complex in mitochondria.

The murine monoclonal antibody chosen for this study ($7B_3$) specifically recognizes the α subunit of F_1 [30] and binds very efficiently to F_1 integrated in the inner mitochondrial membrane, as shown either by competition experiments [31] or by immunoelectronmicroscopy [32]. Fig. 1 shows that the binding of this antibody to isolated F_1 or to electron-transport particles is very fast. Indeed, the binding follows a biphasic kinetics with rate constants of 0.16 and 0.014 s^{-1} for F_1 or 0.1 and 0.004 s^{-1} for ETP (when these proteins are adsorbed on microtitration plates). The biphasic kinetics can be due either to the fact that the antibodies are bivalent or that there is a heterogeneity of the antigen. Similar biphasic kinetics have been observed with monovalent Fab fragments (results not shown). Therefore, the biphasicity is related to heterogeneity of the antigen. This heterogeneity is either inherent in the structure of F_1 [1,31] or can be induced by the adsorption of the protein on the plates. In any case, the rapidity with which this antibody can bind to F_1 integrated into the inner membrane made it a convenient tool to study its effects on the rate of ATP synthesis.

Fig. 2 shows that the maximal inhibition of ATP synthesis induced by the mAb (IgG) is of about 50%. This inhibition is observed with a concentration of 0.3 μM $7B_3$ (IgG). The inhibition is not further increased even when this concentration reaches 2 μM. The monovalent Fab fragments of $7B_3$ induce a maximal inhibition of 45% when their concentration reaches a value of 0.75 μM (fig. 2). The fact that this maximal inhibition

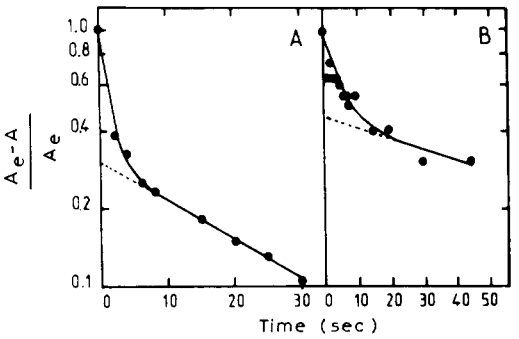

Fig. 1. Rate of mAb binding to F_1 and to electron transport particles. F_1 and electron-transport particles, prepared from pig heart mitochondria, as previously described in Refs. 33 and 34, respectively, were coated to 96-wells microtitration plates, as detailed in Ref. 31. The mAb $7B_3$ [30] purified by the method of Ey et al. [35] was manually added to the wells at a concentration of 1 μM in 50 μl of PBS (10 mM sodium phosphate/150 mM NaCl, pH 7.2) and then removed with a vacuum pump. The time indicated on the abscissa corresponds to the time elapsed between addition and removal of mAb. Addition and removal could be performed at best within 2 s. The wells were immediately washed three times with PBS containing 0.05% Tween 20 (w/v). The amount of mAb bound to the wells was determined by ELISA with an anti-mouse antibody-peroxidase conjugate [30]. Data are plotted according to the equation $\ln (Ae - A/Ae) = -kt$, where Ae = mAb bound at 3 h (maximal binding), A = mAb bound at time t. Each point was made at least in duplicate and the experiments were repeated twice. (A) Wells were coated with 2 μg of F_1. (B) Wells were coated with 13.5 μg of electron-transport particles (containing 2 μg of F_1, as determined in Ref. 36).

is produced by a higher concentration of Fab fragments than that of IgG is likely due to an affinity of the Fab fragments known to be lower than that of IgG for antigens [39]. One can exclude that this high ratio is due to a higher number of moles of Fab fragments bound to F_1 than that of IgG bound to F_1. Indeed, independent experiments [40] have demonstrated that a maximum of two moles of either bivalent IgG or monovalent Fab fragments are bound per mole of F_1 even when the ratio of Fab or IgG to F_1 is increased from 0.3–0.75 to 1.5–2 μM in the incubation medium. The partial inhibition (45–50%) of ATP synthesis is not the result of the presence of F_1 molecules inaccessible to the antibody in 'right-side out' submitochondrial particles. Indeed, the rate of ATP hydrolysis (or ATP synthesis) in the presence or the absence of antibody was not modified by addition of 100 μM carboxyatractyloside. This inhibitor of the adenine nucleotide translocator is known to bind to the outer face of the inner mitochondrial membrane [41] and would inhibit ATP hydrolysis or ATPsynthesis if ATP or ADP should cross the membrane to reach F_1. In conclusion, these experiments show that there is only a very small difference in the maximal inhibition of ATP synthesis induced upon binding of two Fab fragments corresponding to a mass of about 100 kDa or of two IgG corresponding to a mass of about 300 kDa.

To test whether the rate of ATP synthesis could be further diminished by increasing the load on F_1, a polyclonal antibody directed against mouse immunoglobulins was added to the $7B_3$-F_1 complex. Under these conditions, the mass of antibodies bound to F_1 is greatly increased up to about 900 kDa. Indeed, anti-mouse IgG were added in a

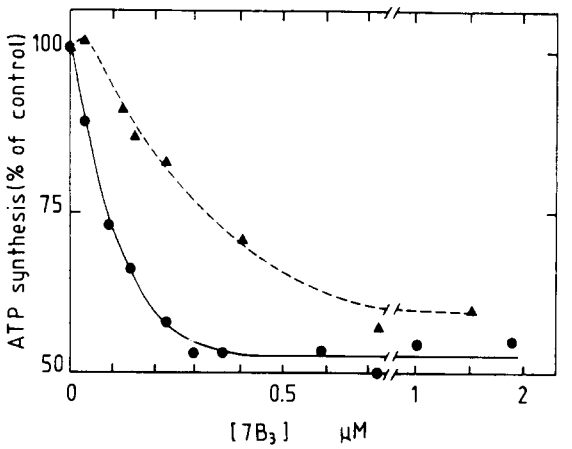

Fig. 2. Inhibition of sucicnate-driven ATP synthesis in electron transport particles upon binding of various concentrations of the mAb, $7B_3$. Various concentrations of IgG (●) or Fab (▲) fragments purified as reported in Refs. 35 or 37, respectively, were incubated for 6 min at room temperature with electron-transport particles (0.37 mg of protein per ml of buffer: 0.25 M sucrose/42 mM sodium phosphate, pH 7.5). Samples containing 75 μg of ETP were then introduced into 0.8 ml of the ATP synthesis medium: 0.25 mM sucrose/50 mM Tris-acetate (pH 7.5)/50 mM glucose/20 mM potassium phosphate $+2 \cdot 10^6$ cpm of [$^{32}P_i$] per μmol of P_i/5 mM $MgCl_2$/40 units of hexokinase per ml/10 mM succinate (final concentrations). After 4 min at 30 °C, the reaction was started by the addition of 1 mM ADP and stopped 5 min later by injection of 0.6 ml of the assay into a P_i extraction medium, as previously described [38]. The rate of ATP synthesis measured in the absence of mAb was 200 nmol ATP synthesized/min per mg protein.

TABLE I

RATE OF ATP SYNTHESIS IN ELECTRON-TRANSPORT PARTICLES AFTER FORMATION OF ANTIGEN-ANTIBODY COMPLEXES OF VARIOUS SIZE

Experimental conditions were the following: (a) Preincubation of electron-transport particles with the antibodies. Electron-transport particles (200 μg) were incubated for 10 min at room temperature with 45 μg of either purified mAb $7B_3$ (IgG) or mouse IgG, or with 20 μg Fab fragments in 42 μl of 60 mM sucrose and 0.1 M sodium phosphate (pH 8.0). This 10-min incubation was sufficient to bind the mAb to F_1 present in the electron-transport particles. (b) Second incubation in the presence or absence of anti-mouse IgG. Where indicated, anti-mouse IgG were then added in a ratio of 2 mol anti-IgG per mol IgG, in a final volume of 82 μl containing 30 mM sucrose and 0.125 M sodium phosphate (pH 8.0). This incubation was made at room temperature for 30 min in order to permit the binding of the anti-IgG to the $7B_3$ (IgG) previously bound to F_1. (c) Measurement of ATP synthesis. 30 μl-samples of each assay were introduced into the succinate-driven ATP synthesis reaction mixture. The rate of ATP synthesis was measured as described in Fig. 2.

Addition	ATP synthesis	
	(nmol ATP/min per mg protein)	(% inhibition)
No addition	263	0
$7B_3$ (Fab fragments)	144	45
$7B_3$ (IgG)	138	48
$7B_3$ (IgG) + anti-mouse IgG	140	47
Mouse IgG	268	0
Mouse IgG + anti-mouse IgG	233	12

ratio of 2 anti-mouse IgG per mol $7B_3$. Therefore, there are 2 IgG-$7B_3$ (300 kDa) + 4 anti-mouse IgG (600 kDa) bound per F_1. The presence of these anti-mouse antibodies did not increase the $7B_3$-induced inhibition of the ATP synthesis rate at all (Table I). It was checked that the presence of either a mouse IgG or mouse IgG-anti-mouse IgG complex had no significant effect on the rate of ATP synthesis.

In conclusion, there is no change in the rate of ATP synthesis when the mass of antibody bound to F_1 varies between 100 and 900 kDa. If the rotation of the α and β subunits were an essential feature of the ATP synthesis mechanism, as proposed by Boyer [19], an increase of the load carried by the α subunit should either completely inhibit or at least decrease the rate of ATP synthesis in a way proportional to the size of the load. The experiments described in this paper demonstrate that this is not the case. Therefore, a mechanism of ATP synthesis in which F_1 should rotate around F_0 or around the minor subunits during catalysis is extremely unlikely. These experiments do not, however, completely exclude a possible rotation of some subunits of F_0 inside the membrane during proton translocation coupled to ATP synthesis, as proposed by Cox [21]. However this hypothesis remains to be demonstrated.

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